# Antioxidant activity of *Nelumbo nucifera* (Gaertn) flowers in isolated perfused rat kidney

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**RESUMO: "Atividade antioxidante das flores de *Nelumbo nucifera* (Gaertn) em rim isolado perfundido de rato".** A padronização da indução de estresse oxidativo com a mstura de Fenton em rim isolado perfundido de rato e a avaliação do efeito antioxidante das flores de *Nelumbo nucifera* em rim isolado de rato oxidativamente estressado são realizadas no presente estudo. Seis grupos de rim isolado perfundido de rato foram utilizados para o presente estudo. O grupo I de rins recebeu veículo. O grupo II de rins foi oxidativamente estressados com a mistura de Fenton. Os grupos III e IV de animais foram tratados com duas doses graduais de extrato após a administração da mistura de Fenton. Os grupos V e VI de rins foram perfundidos com duas doses graduais de extrato sem a mistura de Fenton. A mistura de Fenton causou estresse oxidativo com diferença significativa de malondialdeído (TBARS), glutationa reduzida (GSH), glutationa peroxidase (GPx), catalase, glutamato oxaloacetato transaminase (GOT) e glutamato piruvato transaminase (GPT) ($p<0,05$) em ratos doentes. No tratamento dos animais com extrato, o estresse oxidativo foi diminuído com aumento nos antioxidantes e as enzimas marcadoras mantiveram o nível normal. Este efeito do extrato foi dependente da dose. Em conclusão, a mistura de Fenton desenvolveu o estresse oxidativo em rim isolado perfundido de rato e o extrato das flores de *Nelumbo nucifera* apresenta atividade antioxidante no rim isolado perfundido oxidativamente estressado.

**Unitermos:** *Nelumbo nucifera*, Nymphaceae, mistura de Fenton, malondialdeído, GOT, rim isolado perfundido.

**ABSTRACT:** Standardization of induction of oxidative stress with Fenton mixture in isolated perfused rat kidney and evaluation of antioxidant effect of *Nelumbo nucifera* flowers in isolated oxidatively stressed rat kidney are carried out in present study. Six groups of isolated perfused rat kidney were used for the present study. Group I kidneys were received vehicle. Group II kidneys were oxidatively stressed with Fenton mixture. Group III and IV animals were treated with two graded doses of extract after the administration of Fenton mixture. Group V and VI kidneys were perfused with two graded doses of extract without Fenton mixture. Fenton mixture was found to cause oxidative stress with significant difference of malondialdehyde (TBARS), Reduced glutathione (GSH), Glutathione peroxidase (GPx), catalase, Glutamate Oxaloacetate Transaminase (GOT) and Glutamate Pyruvate Transaminase (GPT) ($p<0.05$) in diseased rats. On treating animals with extract, the oxidative stress was decreased with increase in antioxidants and the marker enzymes were found to maintain the normal level. This effect of extract was found to be dose dependent. In conclusion, Fenton mixture developed the oxidative stress in isolated perfused rat kidney and *Nelumbo nucifera* flowers extract exhibits antioxidant activity in that oxidatively stressed isolated perfused kidney.

**Keywords:** *Nelumbo nucifera*, Nymphaceae, Fenton mixture, malondialdehyde, GOT, isolated perfused kidney.

## INTRODUCTION

Reactive oxygen species (ROS) are generally considered as toxic molecules, and are often associated with tissue injury. Free radicals are molecular species possessing one or more unpaired electrons in their outer orbits. Hydrogen peroxide ($H_2O_2$), because of the absence of unpaired electron, is referred to as ROS and not as a free radical. Presence of unpaired electron configuration imparts greater reactivity to the molecule. A handful of reactions comprise the essentials of free radical chemistry (Halliwell and Gutteridge, 1984).

The chemical reactivity of reactive oxygen

species extends to lipids, carbohydrates, protein and nucleic acids. Free radicals such as hydroxyl ions are capable of initiating chain reactions in lipid domains that result in lipid peroxidation. Peroxidation of membrane lipids perturbs membrane fluidity, permeability, ion and solute transport. Reactive oxygen species also impair enzymes and structural protein molecules through such mechanisms as oxidation of sulfhydryl group and deamination. Additionally, $H_2O_2$ compromises mitochondrial ATP synthesis by inhibiting the ATPase-synthase complex. These changes are followed by the elevation in intracellular calcium, disruption of the cytoskeleton, blebbing of the plasma membrane, and finally cell lysis. Thus lipid peroxidation plays an important role in oxidant -induced early cell injury (Salahydden, 1995).

The main source of hydroxyl radicals is Haber-Weiss reaction, where superoxide radical reduces $Fe^{3+}$ to $Fe^{2+}$ and in that way initiates the Fenton reaction between $Fe^{2+}$ and $H_2O_2$ (Kehrer, 2000).

$$O_2^{\bullet -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \bullet OH$$
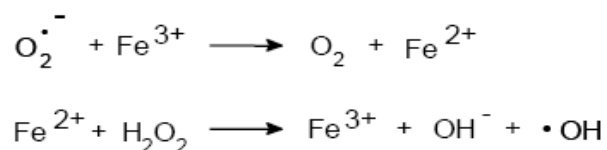
The iron is oxidized to $Fe^{3+}$ and becomes inactive for further reaction. In a chemical Fenton system, a reducing agent such as ascorbic acid is usually added to regenerate $Fe^{2+}$.

*Nelumbo nucifera* (Gaertn) is belongs to the family Nymphaceae. *Nelumbo nucifera* is a native of China, Japan and possibly India. The sacred water lotus has been used in the indigenous system of medicine. All parts of the plant are used. They are astringent, cardio tonic, febrifuge, hypotensive, resolvent, stomachic, styptic, tonic and vasodilator. Rai et al. (2006) mentioned the anti-diarrheal, psychopharmacological, diuretic, antipyretic, antimicrobial, hypoglycemic activity of *Nelumbo nucifera*. A decoction of the flowers is used in the treatment of premature ejaculation (Duke et al., 1985). A decoction of the floral receptacle is used in the treatment of abdominal cramps, bloody discharges etc (Duke et al., 1985). Antioxidant activity of various parts of *Nelumbo nucifera* is well established, e.g. leaf (Wu et al., 2003), stamens and rhizomes (Hu and Skibsted, 2002).

Large number of references has mentioned the antioxidant activity of different parts of *Nelumbo nucifera* in *in-vitro* studies (Phonkot et al., 2008). However the antioxidant activity of flowers has not been scientifically proved. This made us to evaluate the antioxidant activity of *Nelumbo nucifera* flowers in oxidative stress induced in isolated perfused kidney by Fenton mixture. This is the first study which evaluates the oxidative stress induced in isolated perfused rat kidney with Fenton mixture.

## MATERIAL AND METHODS

### Collection and identification of flowers

Flowers of *Nelumbo nucifera* were collected from Thovalai, Nagercoil District, Tamilnadu State, India. The plant materials were identified and authenticated at Division of Pharmacognosy, Centre for Advanced Research in Indian System of Medicine (CARISM), Sastra University, Thanjavur, Tamilnadu, India. The specimen of the same is maintained in the department and the voucher number is 0092.

### Extraction of plant material

The collected flower part was dried under shade for one week and coarsely powdered. The powdered plant material was then extracted with 70% ethanol by cold percolation method. The extract was concentrated *in-vaccuo*. The brown colored residue was used for the following experiment. The extract was dissolved in physiological salt solution (PSS).

### Perfusion apparatus (Morgan et al., 1961)

In this system the kidney was suspended in a 2 x 20 cm (internal dimensions) water-jacketed chamber with a coarse sintered glass filter disk sealed into the lower portion. A mixture of moistened $O_2$:$CO_2$ (95:5) was delivered by small diameter tubing to the lower portion of the chamber by the aerator. An 18 -gauge hypodermic needle, approximately 2.5 cm long, was used as the arterial cannula. The perforate entered the renal circulation by way of the cannulated renal artery, and on leaving the kidney, flowed down the surface of the chamber and through the filter. Satisfactory pressure and flow rate were obtained in the subsequent perfusion by making a preliminary adjustment of the reservoir to produce appropriate pressure. This device permits perfusion by gravity flow. This chamber was at a height of approximately 76 cm above the kidney.

### Perfusate composition

A modified Krebs-Henseleit bicarbonate buffer (Nishiitsutsuji-uwo et al., 1967) (pH 7.4; temperature, 38 ºC) was prepared to obtain the following final concentrations: 118 mMol sodium chloride, 4.7 mMol potassium chloride, 2.4 mMol calcium chloride, 1.2 mMol potassium phosphate, 0.5 mMol calcium-EDTA, 11 mMol glucose, 25 mMol Sodium hydrogen carbonate, and 1.2 mMol magnesium sulfate. The perfusion medium was filtered successively through filters, prior to use and was equilibrated with mixture of $O_2$: $CO_2$ (95:5) at 37 ˚C for 1 h before and throughout the perfusion.

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## Preparation of Fenton mixture

The method of preparation of Fenton mixture is similar to that reported earlier (Venkatesham et al., 2005). About 13.9 mg $FeSO_4.7 H_2O$, 75 mg of sodium EDTA, and 30 µl of 50% $H_2O_2$ were added to 10 ml of 0.1 M dipotassium hydrogen orthophosphate solution and the reaction mixture was kept in a water bath at 40 °C for 20 minutes. With continuous stirring and the solution was used as a source of hydroxyl free radical.

## Operative procedure (Nishiitsutsuji-uwo et al., 1967)

Albino Wistar rats weighing 350 to 450 g were anaesthetized by an intraperitoneal injection of sodium pentobarbitone (40 mg $kg^{-1}$). A midline laparotomy incision was made from pelvis to sternum on the animal. The right kidney was used for perfusion, because the mesenteric artery arises from the aorta at the same level as the right renal artery and a cannula can be passed from one to the other without blood loss and without stopping the blood flow to the kidney. To expose the major abdominal vessels and the right kidney, fat and peri-vascular tissues were cleared away by blunt dissection. The adrenal branch of the right renal artery was tied, and loose ligatures were placed around the following blood vessels: (1) inferior vena cava, just below the liver; (2) aorta, above the mesenteric artery; (3) mesenteric artery, near the aorta; (4) mesenteric artery, further from the aorta; (5) right renal artery, at its origin from the aorta; (6) inferior vena cava, between the left and right renal vein; (7) inferior vena cava, below the left renal vein; (8) inferior vena cava, more distally still; (9) left renal vein.

Then heparin (2 ml, 200 units) was injected into the lower inferior vena cava; afterwards the opening in the wall of the vein was then closed by means of a ligature passed over the point of the injecting needle.

## Cannulation

The right renal artery was cannulated via the mesenteric artery. An 18 - gauge hypodermic needle, approximately 2.5 cm long, was used as the arterial cannula. The end was slightly beveled, with the edges filed to produce a smooth tip. As the cannula was pushed into the renal artery, a hemostat holding back washout perfusate was released and perfusion was begun; there was immediate clearing of blood from the kidney. The inferior vena cava was rapidly cut-off and the kidney was completely freed from the animal. The kidney was trimmed of adhering tissue, and, after turning the stopcock to permit circulation of the perfusate, the kidney was suspended within a thermostatically controlled cabinet at 34-37 °C.

## Grouping of kidneys

The kidneys were randomly assigned to the following treatment groups:

Group I - Normal - 15 ml of PSS + 50 ml of PSS + 50 ml of PSS

Group II - Control - 15 ml of PSS + 50 ml FM + 50 ml of PSS

Group III - 15 ml of PSS + 50 ml FM + 50 ml of drug solution (250 mg $kg^{-1}$ b.wt)

Group IV - 15 ml of PSS + 50 ml FM + 50 ml of drug solution (500 mg $kg^{-1}$ b.wt)

Group V - 15 ml of PSS + 50 ml of PSS + 50 ml of drug solution (250 mg $kg^{-1}$ b.wt.)

Group VI - 15 ml of PSS + 50 ml of PSS + 50 ml of drug solution (500 mg $kg^{-1}$ b.wt.)

The experiment was carried out after getting approval from Institutional animal ethical committee. Registration number is 817/04/ac/CPCSEA and the ethical committee clearance number is 7/SASTRA/IAEC/RPP.

## Biochemical parameters

After completion of perfusion, the kidneys were homogenized in ice cold 0.1M 10% Tris HCl buffer (pH 7.4). The homogenate was centrifuged at 4 °C at 1500 rpm for 10 min. The supernatant was used for the estimation of lipid peroxidation (Okhawa et al., 1979), catalase (Sinha, 1972), Glutathione peroxidase (GPx) (Wendel, 1981), reduced glutathione (GSH) (Ellman, 1959), protein (Lowry et al., 1951), glutamate pyruvate transaminase (GPT) (Reitman and Frankel, 1957), glutamate oxaloacetate transaminase (GOT) (Reitman and Frankel, 1957).

## Statistical analysis

Statistical analysis was carried out using SPSS software version 12.0. Values mentioned in the Tables are Mean ± SD. Significant difference was evaluated using One Way ANOVA with Duncan Multiple Range Test (DMRT). $p < 0.05$ was considered as significant difference.

## RESULTS

TBARS was found to be increased in diseased kidney against normal kidney ($p < 0.05$, Table 1). But on treating animals with extract the TBARS level was found to be decreased significantly and dose dependently ($p < 0.05$, Table 1). However, no much significant differences in TBARS level of Group V and VI animals were observed.

The catalase level of diseased kidney was found to be increased in diseased kidney against normal kidney ($p < 0.05$, Table 1). But it was found to be decreased in treatment dose dependently and significantly ($p < 0.05$,

Table 1). No significant difference had been observed in plant control animals.

GPx and GSH level of diseased kidney was found to be decreased against normal kidney (p<0.05, Table 1). Extract treatment was found to increase the level of both GSH and GPx significantly and dose dependently (p<0.05, Table 1). No significant difference in GSH and GPx level was observed.

GOT and GPT level was found to be increased in diseased kidney against normal kidney (p<0.05, Table 2). Treatment was found to decrease the level of both the enzymes and return back both the enzymes level to the normal level.

## DISCUSSION

Iron, an essential element is important for metabolic functions such as oxygen transport and enzymatic reactions. The free iron catalyzed the generation of hydroxyl radicals from superoxide and $H_2O_2$ via Fenton reaction. These hydroxyl radicals subsequently cause lipid peroxidation (Toyokuni, 1996). The externally administered Fenton mixture was responsible for generating a large number of hydroxyl radical and free iron. Moreover, superoxide radical generated by the oxidative stress increases the level of

free iron ions, and also acting as a reducing agent in the Fenton reaction (Gardner, 1997). Leake and Rankin, (1990) have mentioned that iron could accumulate along the negatively charged lipid bilayer and subsequently caused membrane damage. The ferrous state of iron is free to participate in Fenton chemistry and produce the hydroxyl free radical which accelerated the lipid oxidation by breaking down hydrogen and lipid peroxides (Dunford, 1987). These findings were agreed with our observations. These reasons might be responsible for significantly increased TBARS level in diseased kidneys (Group II, p<0.05, Table 1) against normal kidney.

On treating kidneys with *Nelumbo nucifera* extract alone, no much significant difference in TBARS was observed. These results showed that the *Nelumbo nucifera* was not able to generate lipid peroxidation by its own. But treating kidney with extract prevented the much production of TBARS (Group III & IV). The decrement of TBARS observed in treatment was dose dependently and significantly (p<0.05). This might be due to the antioxidant or free radical scavenging effect of *Nelumbo nucifera*. The antioxidant activity of leaves of *Nelumbo nucifera* had been reported earlier by Saengkhae (Saengkhae et al., 2007).

As a product of lipid peroxidation, superoxide radicals were generated which later converted into

**Table 1.** Effect of *Nelumbo nucifera* flowers on antioxidants of kidney homogenate.

| Treatment | TBARS (nM of MDA mg of protein$^{-1}$) | Catalase (nM of $H_2O_2$ used min $^{-1}$mg of protein $^{-1}$) | GPx (nM of GSH used min $^{-1}$ of protein $^{-1}$) | GSH (nMOL of GSH mg of mg protein $^{-1}$) |
|---|---|---|---|---|
| Group I | 0.28 ± 0.01* | 58.9 ± 3.1* | 1.18 ± 0.06* | 25.1 ± 1.0 * |
| Group II | 2.1 ± 1.5*** | 80.3 ± 23.5*** | 0.97 ± 0.3*** | 18.9 ± 5.8*** |
| Group III | 0.53 ± 0.1** | 69.01 ± 3.1** | 0.93 ± 0.1** | 18.4 ± 4.1*** |
| Group IV | 0.25 ± 0.2* | 57.1 ± 4.7* | 1.1 ± 0.09* | 22.2 ± 4.04** |
| Group V | 0.27 ± 0.05* | 56.9 ± 4.1 * | 1.16 ± 0.11* | 24.3 ± 0.82* |
| Group VI | 0.25 ± 0.09* | 57.2 ± 1.2 * | 1.17 ± 0.02* | 24.17 ± 1.7* |

Values are Mean ± SD. Values not sharing common number of * (for eg *, **, ***) are differ significantly at p<0.05.

**Table 2.** Effect of *Nelumbo nucifera* flowers on kidney markers.

| Treatment | GPT (μMol of pyruvate liberated min$^{-1}$ mg of protein $^{-1}$) | GOT (μMol of pyruvate liberated min$^{-1}$ mg of protein$^{-1}$) |
|---|---|---|
| Group I | 5.32 ± 0.37 * | 5.8±0.35* |
| Group II | 8.3 ± 2.2 ** | 9.66±3.2** |
| Group III | 5.65 ± 0.76 * | 5.74±0.67* |
| Group IV | 5.30 ± 0.56 * | 5.61±0.73* |
| Group V | 5.32 ± 0.57* | 5.92±0.52* |
| Group VI | 5.01 ± 0.18* | 5.86±0.22 * |

Values are Mean ± SD. Values not sharing common number of * (for eg *, **, ***) are differ significantly at p<0.05.

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$H_2O_2$ by the enzyme superoxide dismutase. Qing et al. (2004) have explained that lower concentration of $H_2O_2$ was non-enzymatically converted in an endoplasmic reticulum based Fenton reaction. Higher concentration of $H_2O_2$ levels were controlled mainly by GPx in cytosol and mitochondria or by catalase in peroxisomes. Administration of large amount of $H_2O_2$ in diseased kidney might be responsible for the decreased level of GPx (p<0.05, Table 1). On treating animals with extract the GPx level was found to be increased significantly and dose dependently (p<0.05, Table 1). This effect might be due to the free radical scavenging activity of *Nelumbo nucifera*. Si Eun et al. (2003) have reported that the crude extract of *Nelumbo nucifera* inhibited the $H_2O_2$ induced apoptosis of Chinese hamster lung fibroblast (V79-4) cells and these extract was found to increase the level of GPx.

Moreover, different effect was observed in diseased kidney in the level of catalase. The reason for this increment in catalase level should be further evaluated. Rai et al. (2006) have explained that administration of *Nelumbo nucifera* seeds was found to be increased the level of catalase enzyme dose dependently. But in our present research work the level of enzyme catalase was found to be decreased dose dependently. This might be due to the hydrogen peroxide radical scavenging activity of the extract without increasing the synthesis of the enzyme catalase.

Spear and Aust (1995) have explained that GSH protected DNA from oxidation by Fe (II) and $H_2O_2$. Thus GSH had been used to protect the kidney from oxidative stress resulted in decreased level of GSH in diseased kidney against normal. Earlier references showed that the Fenton reaction mediated DNA oxidation was inhibited by *Nelumbo nucifera* (Wu et al., 2003). In our present result, the concentration of GSH was found to be increased in treated kidney against diseased kidney (p<0.05). This might be due to the ability of *Nelumbo nucifera* to prevent the utilization of GSH.

In another study, which is yet to be published, we have observed that administration of Fenton mixture was found to prevent the beat of isolated perfused heart in *in-vitro* condition. This condition might be responsible for the oxidative stress or necrosis in heart. Similar necrosis might be developed in kidney by administering Fenton mixture. This research work made us to estimate marker enzymes in kidney. In our present study, administration of Fenton mixture caused significantly increased level of GOT and GPT enzymes (p<0.05, Table 2) in diseased kidney against normal kidney.

Gayathri et al. (2007) have mentioned that oxidative stress caused by $CCl_4$ was found to decrease the level of enzymes in kidney. But in our study, GOT and GPT level of kidney was found to be increased significantly (p<0.05, Table 2). Oxidative stress induced pro-inflammation was well described by Rajiv, (2003). Inflammation caused by administration of Fenton mixture

resulted in the oxidative stress might be responsible for this increment.

Sohn et al. (2003) have mentioned that the hepatoprotective effect of *Nelumbo nucifera* might be due to the antioxidant effect. These earlier references and also the results of our present findings strengthen the result that the decreased level of marker enzymes like GOT and GPT observed in treatment with extract might be due to the antioxidant activity of *Nelumbo nucifera*.

## CONCLUSION

In conclusion, Fenton mixture was found to develop the oxidative stress in isolated perfused rat kidney in *in-vitro* condition. Moreover, the 70% ethanol extract of *Nelumbo nucifera* flowers was found to inhibit the damages caused by oxidative stress in kidney.

## ACKNOWLEDGEMENTS

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